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Supplemental Data

Antagonistic Regulation of PIN Phosphorylation by PP2A and PINOID Directs Auxin Flux

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Supplemental Figures

Figure S1. Expression of *PP2AA*s during seedling development.

(A-C) GUS staining shows overlapping expression patterns of *PP2AA1::GUS* (A), *PP2AA2::GUS* (B) and *PP2AA3::GUS* (C) in transgenic seedlings. GUS activity was found predominantly in primary root, lateral roots (with exception of *PP2AA3*), root-shoot junction, cotyledons and shoot apical meristem.

(D-E) Localization of PP2AA1 (*PP2AA1::PP2AA1:GFP*, D) and PID (*PID::PID:YFP*, E) proteins in primary roots.

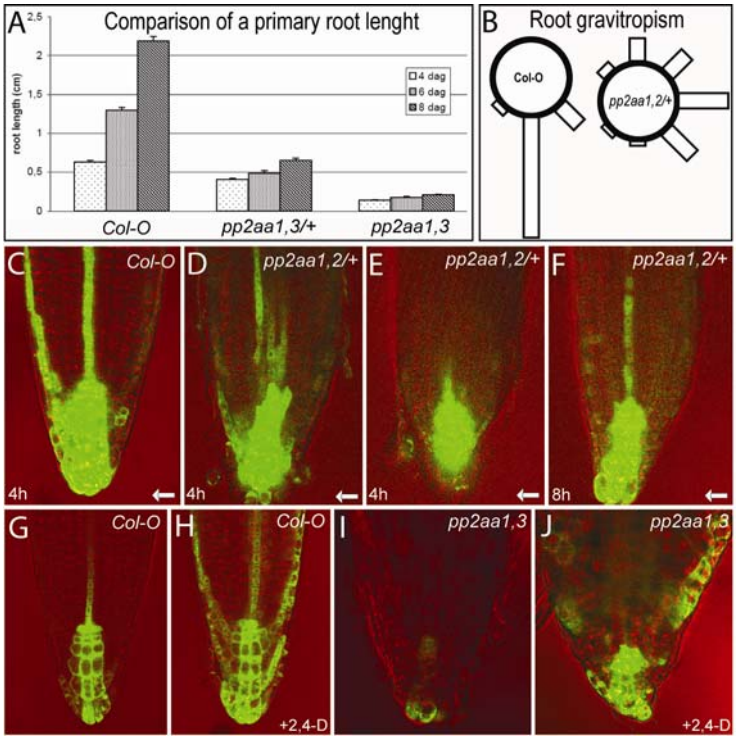

Figure S2. Development and *DR5* activity in *pp2aa* primary roots.

(A, B) Root phenotypes of *pp2aa1 pp2aa2/+*, *pp2aa1 pp2aa3/+* and *pp2aa1 pp2aa3* as compared to wild-type include defects in primary root growth (A) and impaired gravity response (B).

(C-F) Distribution of $DR5_{rev}$*::GFP* expression in gravistimulated roots exhibit no or only weak relocation of *DR5* signal 4 or 8 hours after gravistimulation as compared to wild-type. Whereas all wild-type roots exhibited asymmetric *DR5* activity accumulation along the lower side of roots within 3-4 hours (n = 25) (C), only about 23% (n = 48) of *pp2aa1 pp2aa2/+* mutant roots showed a slight (D) or no (E) relocation of the GFP signal even after 8 hours (F). Arrows indicate direction of gravity vector.

(G-J) Increased *DR5* expression in roots incubated in 2,4-D for 4 hours (H,J) as compared to untreated controls (G,I) in both wild-type (G,H) and *pp2aa1 pp2aa3* (I,J) roots.

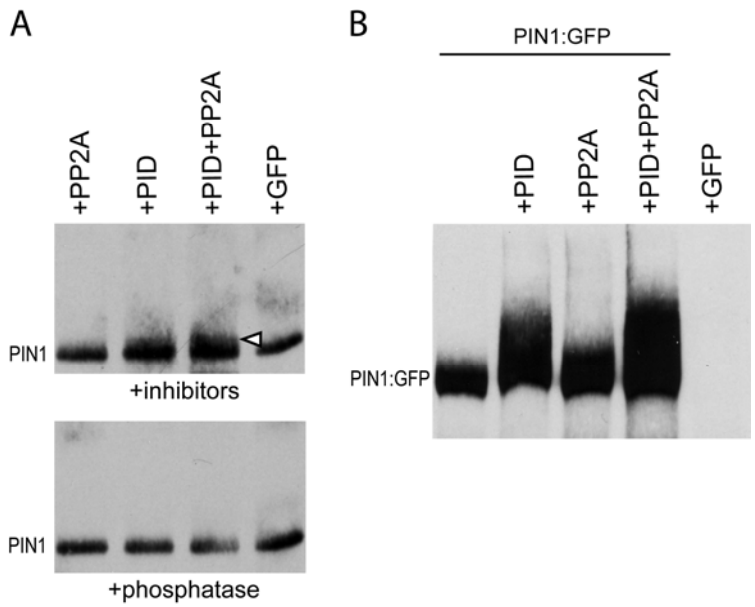

Figure S3. PID-dependent phosphorylation of PIN1 in *Arabidopsis* protoplasts.

(A) When co-transfected with *35S::PID:FLAG* (with or without *35S::PP2AA1:HA*), higher molecular weight bands of endogenous PIN1 (arrowheads) appear. The appearance of additional PIN1 bands is sensitive to λ-phosphatase treatment. This effect of PID on endogenous PIN1 is to large extent masked when co-transfected with *35S::PIN1:GFP* (see Figure 6C). This is due to the low transfection efficiency; only about 10% of endogenous PIN1 that is seen on the blot was derived from successfully transfected cells and thus accessible to any overexpressed PID. The remaining 90% of endogenous PIN1 would be not modified in a PID-dependent manner as it originates from non-transfected cells. In contrast, almost 100% of co-transfected PIN1:GFP originates from PID:FLAG-positive protoplasts. Moreover, similar signal intensities that we observed, when comparing endogenous PIN1 vs. PIN1:GFP, demonstrated much higher abundance of PIN1:GFP in transfected cells. This explains the preferential phosphorylation of PIN1:GFP over endogenous PIN1in these cells.

(B) Phosphorylation assays in *Arabidopsis* protoplasts: Immunoprecipitation of PIN1:GFP by anti-PIN1 antibodies confirms PID-dependent modification of PIN1.

Supplemental Table

Table S1. Sequences of recovered peptides from the PIN1:GFP immunoprecipitation as derived from MS/MS data. Note peptide 5, which was recovered in both phosphorylated and non-phosphorylated form.

No	Peptide	Pos.	z	Score (XC)[a]	Ions (MS/MS)
1	KVLATDGGNNISNKT	435	2	2.91	20/24
2	RPSNYEEDGGPAKPTAAGTAAGAGRF	288	2	4.16	21/46
3	KGPTPRPSNYEEDGGPAKPTAAGTAAGAGRF	283	3	3.78	34/112
4	KISVPQGNSNDNQYVERE	406	2	2.76	17/30
5a	RFHYQSGGSGGGGGAHYPAPNPGMF**pS**PNTGGGGGTAAKG	312	3	3.99[b]	53/216
	RFHYQSGGSGGGGGAHYPAPNPGMFSPN**pT**GGGGGTAAKG			3.82	55/216
5b	RFHYQSGGSGGGGGAHYPAPNPGMFSPNTGGGGGTAAKG	312	3	3.90	37/144
6	RPSNLTNAEIYSLQSSRN	229	2	4.99	22/30
7	RNSNFGPGEAVFGSKG	269	2	3.59	18/26

[a] Cross-correlation (XC) significance thresholds after Peng et al. (2003) are 1.5 and 3.3 for doubly- and triply-charged peptides, respectively.
[b] The MS/MS spectrum of this peptide fits almost equally well with phosphorylation at Serine 337 (3.99) as on Threonine 340 (3.82).

PIN1 protein sequence with recovered peptides highlighted:

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  1 MITAADFYHV MTAMVPLYVA MILAYGSVKW WKIFTPDQCS GINRFVALFA
 51 VPLLSFHFIA ANNPYAMNLR FLAADSLQKV IVLSLLFLWC KLSRNGSLDW
101 TITLFSLSTL PNTLVMGIPL LKGMYGNFSG DLMVQIVVLQ CIIWYTLMLF
151 LFEYRGAKLL ISEQFPDTAG SIVSIHVDSD IMSLDGRQPL ETEAEIKEDG
201 KLHVTVRRSN ASRSDIYSRR SQGLSATPRP SNLTNAEIYS LQSSRNPTPR
251 GSSFNHTDFY SMMASGGGRN SNFGPGEAVF GSKGPTPRPS NYEEDGGPAK
301 PTAAGTAAGA GRFHYQSGGS GGGGGAHYPA PNPGMFSPNT GGGGGTAAKG
351 NAPVVGGKRQ DGNGRDLHMF VWSSSASPVS DVFGGGGGNH HADYSTATND
401 HQKDVKISVP QGNSNDNQYV EREEFSFGNK DDDSKVLATD GGNNISNKTT
451 QAKVMPPTSV MTRLILIMVW RKLIRNPNSY SSLFGITWSL ISFKWNIEMP
501 ALIAKSISIL SDAGLGMAMF SLGLFMALNP RIIACGNRRA AFAAAMRFVV
551 GPAVMLVASY AVGLRGVLLH VAIIQAALPQ GIVPFVFAKE YNVHPDILST
601 AVIFGMLIAL PITLLYYILL GL
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329 aa cytoplasmic loop
137 aa (42%) coverage of the cytoplasmic loop in MS
Transmembrane domains; Peptides recovered in MS.

Supplemental Experimental Procedures

The following mutants and transgenic plants have been described previously: *DR5$_{rev}$::GFP* (Benková et al., 2003), *pp2aa1* (*rcn1)* (Garbers et al., 1996), *35S::PID* and *pid* (EN197) (Benjamins et al, 2001). *PP2AA* promotor::*uidA* (GUS) fusions were generated using approximately 2 kb of At1g25490 (*PP2AA1*), At3g25800 (*PP2AA2*) and At1g13320 (*PP2AA3*) 5'UTRs. The promotor fragments were amplified from genomic DNA by using the following primers combinations: 5'-TCACTTACCAAGCTTCGGATGATCCA-3' and 5'-CGCGGATCCCTTATGTGAAAGTTCGAATCA-3' for *PP2AA1*; 5'-CGCGAGCTCCCTGAGATTGATACATTGA-3' and 5'-CGCGGATCCCTTCAACAACACCAACAAC-3' for *PP2AA2* and 5'-ACGCGTCGACCATCGTATTCAATTCCAAGCTC-3' and 5'-CGGGATCCCCTCACCAAAACTCAAATCACT-3' for *PP2AA3*. Fragments were cloned into pSDM 7006, pVKH-35S-GUS-pA and pCAMBIA-1391Z binary vectors, respectively (Weijers et al., 2003; Hamann et al., 2002; McElroy et al., 1995). The *PP2AA1:GFP* C-terminal translational fusion was created inserting *PP2AA1* genomic fragment between 2035 bp upstream and 3194 bp downstream from ATG into pGreenII Kan-tNOS (Hellens et al., 2000) and its functionality was confirmed in *pp2aa1* and *pp2aa1 pp2aa3* mutants. The *PID:YFP* translational fusion was created by cloning a BamHI fragment containing the complete *PID* promoter plus coding region from *PID:GUS* (Benjamins et al., 2001) upstream and in frame with a 9X poly-alanine linker and the GFP variant VENUS (Nagai et al., 2002). Functionality of the PID:YFP translational fusion was confirmed by transformation into *pid-6 /PID* heterozygotes using the binary vector pMLBART (Eshed et al., 2001). Two independent amiRNAs were engineered according to Schwab, et al., 2006 and placed under the *UAS* promotor. 21 bp oligonucleotides used for PCR were: 5'-TATTGCCCATTCAGGACCGAA-3' for amiRNA-1 and 5'-TTGCATGCAAAGGGCACCGAG-3' for amiRNA-2 construct. The predicted miRNA targets were: At1g25490 (*PP2AA1*), At3g25800 (*PP2AA2*) and At1g13320 (*PP2AA3*). Artificial microRNA fragments were engineered into miR319 and cloned into to pGIIB-UAS-tNOS (Schwab et al., 2006) and the construct was transformed into tamoxifen-inducible pINTAM activator line (Friml et al. 2004). *GST:PID* (Benjamins et al., 2003), pET-*PIN1HL* (amino acids 288-452; Paciorek et al., 2005) and pGEX-*PIN2HL* (Abas et al., 2006) have been described previously. *HIS*:*PID* was created by ligating the *PID* cDNA (Benjamins et al., 2001) into pET16H (pET16B derivative, J. Memelink, unpublished results). *35S::PID:FLAG* was constructed by replacing the BsiWI XhoI 3'

fragment in pSDM70671 (pEF-*PID*; Friml et al., 2004) with a fragment encoding a C-terminal fusion between PID and the FLAG tag and subsequently cloned behind the *35S* promoter. *HIS:MLP* (At5g08120) was obtained by PCR amplification of the cDNA using the primers 5'-ACGCTTGTCGACTATATGTATGAGCAGCAGCAACAT-3' and 5'-CGGGATCCAAACAACCCAAGGAGAGAAATATC-3'. The resulting PCR fragment was cloned into pET16B (Novagen). MBP (myelin basic protein) was purchased from Sigma.

Arabidopsis thaliana (ecotype *Col-0*) plants were transformed into wild-type or pINTAM (amiRNA) activator line. T2 or T3 seedlings for each transgene were identified by antibiotic selection and segregation analysis. T-DNA insertion lines were obtained from NASC: SALK_042724 (*pp2aa2-1)* and SALK_017541 (*pp2aa2-3*); SALK_014113 (*pp2aa3-1*) and SALK_099550 (*pp2aa3-2*). Genotypes of all insertion lines were confirmed by PCR and further analyzed by RT-PCR.

Supplemental References

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wiśniewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J. and Luschnig, C. (2006). Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat Cell Biol. 8, 249-56.

Benjamins, R., Ampudia, C.S., Hooykaas, P.J. and Offringa, R. (2003). PINOID-mediated signaling involves calcium-binding proteins. Plant Physiol. 132, 1623-30.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P. and Offringa, R. (2001). The PINOID protein kinase regulates organ development in Arabidopsis by enhancing polar auxin transport. Development 128, 4057-4067.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G. and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115, 591-602.

Eshed, Y., Baum, S.F., Perea, J.V., Bowman, J.L. (2001). Establishment of polarity in lateral organs of plants. Curr. Biol. 11(6):1251-60.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G., Hooykaas, P.J., Palme, K. and Offringa, R. (2004). A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science 306, 862-865.

Garbers, Ch., DeLong, A., Deruére, J., Bernasconi, P. and Söll, D. (1996). A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in Arabidopsis. EMBO J. 15, 2115-2124.

Hamann, T., Benkova, E., Baurle, I., Kientz, M. and Jurgens, G. (2002). The Arabidopsis BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. Genes Dev. 2002 16(13):1610-5.

Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M. (2000) "pGreen: a versatile and flexible binary Ti vector for Agrobacterium mediated plant transformation", Plant Mol. Bio. 42: 819-832.

McElroy, D., Chamberlain, D. A., Moon, E. and Wilson, K. J. (1995). Development of gusA reporter gene constructs for cereal transformation: Availability of plant transformation vectors from the CAMBIA molecular genetic resource service. Mol. Breed. 1, 27-37.

Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat. Biotechnol. 20(1):28-9.

Paciorek,T., Zazimalova, E., Ruthardt, N., Petrášek, J., Stierhof, Y.D., Kleine-Vehn, J., Morris, D.A., Emans, N., Jurgens, G., Geldner, N. and Friml, J. (2005). Auxin inhibits endocytosis and promotes its own efflux from cells. Nature 435, 1251-1256.

Peng, J., Elias, J.E., Thoreen, C.C., Licklider, L.J. and Gygi, S.P. (2003). Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. J. Proteome Res. 2, 43-50.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N. and Weigel, D. (2006). Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell 18, 1121-1133.

Weijers, D., Van Hamburg, J.P., Van Rijn, E., Hooykaas, P.J. and Offringa, R. (2003). Diphtheria toxin-mediated cell ablation reveals interregional communication during Arabidopsis seed development. Plant Physiol. 133(4):1882-92.